

# An inverse relationship between receptor internalization and the fraction of laterally mobile receptors for the vasopressin renal-type V<sub>2</sub>-receptor

## An active role for receptor immobilization in down-regulation?

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Lateral mobility of the vasopressin renal-type V<sub>2</sub>-receptor was investigated in LLC-PK<sub>1</sub> porcine epithelial cells using the technique of fluorescence microphotolysis (photobleaching) and a rhodamine-labelled vasopressin analogue. At various times after ligand addition, cells were analyzed for both receptor lateral mobility and ligand internalization. The V<sub>2</sub>-receptor mobile fraction diminished from 0.9 to 0.43 over 60 min at 37°C, whereas the apparent lateral diffusion coefficient remained essentially unchanged ( $2-3 \times 10^{-10}$  cm<sup>2</sup>/s). Interestingly, the fraction of immobile V<sub>2</sub>-receptors corresponded exactly with the fraction of internalized receptors, implying a functional relationship. These observations together with comparable results reported for other polypeptide hormone receptors indicate a possible mechanistic role for receptor immobilization in the desensitization of hormonal response.

Vasopressin renal V<sub>2</sub>-type receptor; Photobleaching; Internalization; Lateral mobility

### 1. INTRODUCTION

Lateral mobility of receptors has been proposed to be a determining factor in hormone-mediated signal transduction [1–4]. Models of receptor-mediated activation of adenylate cyclase assert that lateral diffusion of the hormone-receptor complex in the plasma membrane lipid bilayer is the mechanism by which adenylate cyclase comes into contact with, and is activated by the ligand-receptor complex [1]. We have reported a temperature dependence of the fraction of mobile vasopressin renal-type V<sub>2</sub>-receptors, with the highest (100%) mobility at 37°C [5], which argues for a physiological role of receptor lateral diffusion in cyclase-mediated signal transduction. Data from the  $\beta$ -adrenergic receptor-adenylate cyclase [6,7] and other [8–10] systems imply that agonist binding to receptor promotes redistribution of the signal-transduction-mediating GTP (G-) binding G<sub>s</sub> component into the cytosol. This implies that receptor lateral diffusion-mediated interaction with G-proteins may be the rate-limiting step in signal transduction, all subsequent events occurring rapidly in the aqueous phase [11,12].

Direct measurements of receptor lateral mobility using the technique of fluorescence microphotolysis (photobleaching) for the vasopressin vascular-type V<sub>1</sub>- and epidermal growth factor receptors yield a relatively low receptor mobile fraction at 37°C [13–16]. Rather than questioning a physiological role for receptor

lateral mobility, however, this may reflect the fact that rapid receptor internalization hinders measurement of a high receptor mobile fraction due to the time-consuming nature of the photobleaching measurements [13–15,17]. To investigate the role of internalization in receptor immobilization, we chose to reexamine V<sub>2</sub>-receptor lateral mobility at different times after ligand addition, and observed a negative correlation between receptor mobile fraction and internalized receptor.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

Materials were from the sources described previously [5,13]. The rhodamine-labelled analogue of vasopressin, 1-deamino[8-lysine (*N*<sup>6</sup>-tetramethylrhodamylaminothiocarbonyl)] vasopressin (TR-LVP) was prepared as described [5].

#### 2.2. Cell culture

Cells of the LLC-PK<sub>1</sub> porcine kidney epithelial cell line [18] were cultured in DMEM supplemented with 10% (v/v) foetal calf serum, 0.2 mg/ml streptomycin and 50 U/ml penicillin, as previously described [5].

#### 2.3. Fluorescence measurements

Cells to be used for fluorescence and lateral diffusion measurements were grown on coverslips (15 × 15 mm) for 3–4 days to about 50% confluence. After incubation with ligand for various times, cells were washed with NaCl/P<sub>i</sub> (containing 0.5 mg/ml BSA), and mounted in the incubation medium, in the absence of ligand. The instrumentation and procedures used in the measurement of fluorescence intensity and lateral mobility have been described previously in detail [5,19,20]. Fluorescence was monitored at short intervals of the fluorescence recovery process, rather than in the usual continuous fashion, to avoid (unintentional) bleaching during the course of the measurements [5,13].

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#### 2.4. $V_2$ -receptor internalization

[ $^3$ H]Arg-vasopressin internalization was measured in cell monolayers as described previously [5,13], whereby cells were incubated with 20 mM Gly-HCl, pH 3.0, containing 200 mM NaCl for 3 min at 4°C to dissociate surface-bound ligand. Both internalized (pH 3-resistant) and medium-released radioactivity were determined [5]. The contribution of non-specific binding, determined by treating cells as above subsequent to incubation with radioactive ligand together with a 100-fold excess of unlabelled Arg<sup>8</sup>-vasopressin (AVP), was less than 15%. Internalized ligand is expressed as a fraction of total specifically bound ligand.

### 3. RESULTS

Receptor aggregation and internalization has been visualized for the EGF- [15,21] and vasopressin  $V_1$ - [13] receptors, both of which exhibit rapid internalization kinetics ( $t_{1/2}$  = 6 and 2 min, respectively, at 37°C). The  $V_2$ -receptor exhibits much slower ligand internalization ( $t_{1/2}$  = 14 min at 37°C) [5]. The  $V_2$ -receptor was fluorescently labelled by incubating LLC-PK<sub>1</sub> cells with  $10^{-7}$  M TR-LVP either at 4°C for 60 min (in the absence of internalization) or at 37°C for 30 min, prior to washing and mounting in ligand-free medium. Labelling was then visualized by video-enhanced fluorescence microscopy (Fig. 1). Specificity of the labelling was demonstrated by incubating cells with TR-LVP in the presence of  $10^{-5}$  M AVP (Fig. 1C,D,G,H). Cells labelled at 4°C showed a relatively homogeneous plasma membrane labelling (Fig. 1F). This staining was not dissimilar to that obtained with a plasma membrane lipid probe DiOC<sub>14</sub> [3] (not shown). Cells incubated at 37°C, in contrast, showed prevalent fluorescent aggregates (Fig. 1B), which increased in intensity with time at 37°C (not shown) and were localized in the periphery of the nucleus. Comparable results have been observed for the  $V_1$ -receptor [13] as well as for the EGF-receptor [14,15,17,21]. An interesting hypothesis is that the highly fluorescent perinuclear aggregates visualized may be coated pits [22].

To measure lateral mobility of the  $V_2$ -receptor at various times after ligand addition, LLC-PK<sub>1</sub> cells were incubated for 10, 30 and 60 min at 37°C with  $10^{-7}$  M AVP, prior to washing, mounting, and lateral diffusion measurements. Maintaining measurement parameters constant, parallel series of measurements were performed on several different cells for each experimental treatment, with measurements for each time interval then pooled and averaged. Specific fluorescence was calculated by subtracting the values for non-specifically bound fluorescence from those for total bound fluorescence [5,13]. The photobleaching recovery of specific fluorescence was evaluated for two components; a mobile fraction  $f$ , with apparent diffusion coefficient,  $D$ , and an 'immobile' fraction ( $D < 10^{-12}$  cm<sup>2</sup>/s) [23], and data fitted by theoretical curves [24].

The results are depicted in Fig. 2, and summarized in Table I. A marked reduction of  $f$  with time of incubation with ligand (0.91 to 0.43) is evident over 60 min at

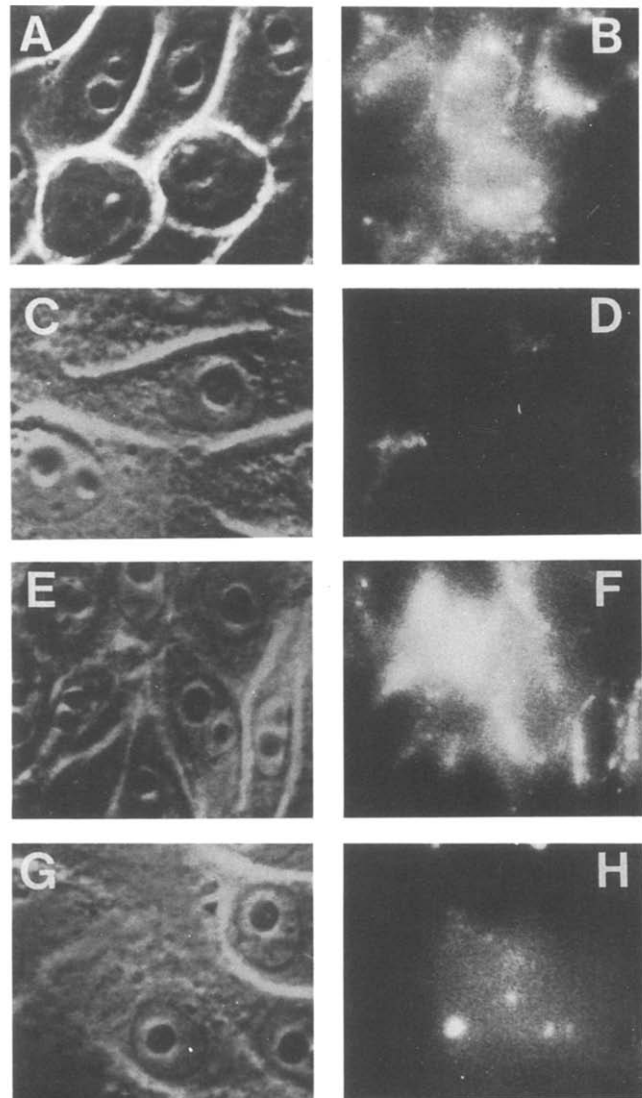


Fig.1. Visualization of binding of TR-LVP to LLC-PK<sub>1</sub> cells. Cells were incubated with TR-LVP ( $10^{-7}$  M) at 37°C for 30 min (A-D) or 4°C for 60 min (E-H), in the absence (A, B, E, F) or presence (non-specific binding) (C, D, G, H) of  $10^{-5}$  M AVP. Cells were then washed, mounted in serum-free medium at 37°C, and photographed using a 100× oil immersion objective under normal (A, C, E, G) or fluorescent (B, D, F, H) illumination.

37°C.  $D$  was only slightly reduced over the same time period ( $2.75$  to  $2.0 \times 10^{-10}$  cm<sup>2</sup>/s) indicating that the process of reduction of  $f$  was one of receptor immobilization rather than effects on general membrane fluidity or diffusion rates (Table I).

Table I shows the results for [ $^3$ H]AVP internalization, performed in parallel, expressed as the fraction of internalized compared to total bound ligand. Internalization is maximal after 60 min (64% of total binding). A clear negative correlation between receptor mobile fraction and internalized receptor is evident (Table I).

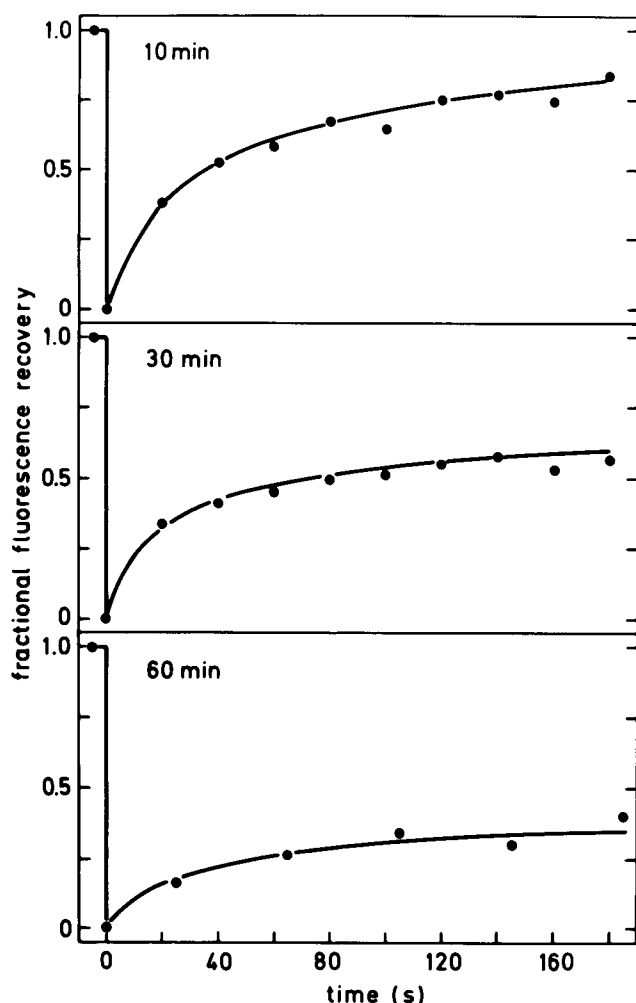


Fig.2. Lateral mobility of the  $V_2$ -receptor in the basal plasma membrane of LLC-PK<sub>1</sub> cells as a function of time after ligand addition. Measurements were performed on cells incubated for the indicated times with  $10^{-7}$  M TR-LVP at 37°C in the absence or presence (non-specific binding) of  $10^{-5}$  M AVP. Cells were then washed, mounted, and photobleaching measurements carried out at 37°C. The curves represent the least square best fits to the experimental data for specific fluorescence (see Table I for pooled data).

#### 4. DISCUSSION

We have argued [5] that because the receptor mobile fraction for the  $V_2$ -receptor is highest at 37°C and lowest at 10°C, receptor lateral mobility in the plasma membrane may be of physiological importance in terms of the mechanism of signal transduction in the adenylate cyclase system. That low  $f$  values at 37°C have been determined for the  $V_1$ - and EGF-receptors may be explained in terms of the rapid internalization kinetics [14,15]. No high  $f$  value can be detected due to the time-consuming nature of the diffusion measurements [13].

Receptor aggregation, immobilization, and internalization occur at a much slower rate in the  $V_2$ -receptor system and here, where the slower internaliza-

Table I  
Lateral mobility of the  $V_2$ -receptor in membranes of LLC-PK<sub>1</sub> cells at different times after ligand addition

Time after incubation with ligand (37°C) <sup>a</sup>	Parameter of mobility <sup>b</sup>			Fraction of internalized receptors <sup>c</sup>
	$D$ ( $10^{-10}$ cm <sup>2</sup> /s)	$f$	$n$	
10 min	$2.75 \pm 0.11$	$0.91 \pm 0.03$	12	0.10
30 min	$3.01 \pm 0.45$	$0.70 \pm 0.06$	6	0.40
60 min	2.00	0.43	2	0.64

<sup>a</sup> Cells were pretreated at 37°C for the indicated times with  $10^{-7}$  M TR-LVP in the absence or presence (non-specific binding) of  $10^{-5}$  M AVP, then washed, mounted, and photobleaching measurements carried out at 37°C

<sup>b</sup> Values are the mean  $\pm$  SE for  $D$ , the apparent lateral diffusion coefficient and  $f$ , receptor mobile fraction, with  $n$  the number of experiments. Each experiment consisted of at least 5 series of single-cell measurements for each of total bound and non-specific fluorescence, respectively. Each single-cell measurement involved monitoring fluorescence over 3 min at 20 or 40 s intervals (see Fig. 2)

<sup>c</sup> Internalized receptor was determined as described in Section 2, using a pH 3 treatment subsequent to the normal [<sup>3</sup>H]AVP binding assay to dissociate non-internalized ligand [5,13]. Both cell associated and medium radioactivity were then determined

tion kinetics permit such an analysis, it was possible to demonstrate that internalization does indeed parallel  $V_2$ -receptor immobilization and reduction of  $f$  (Table I). The hypothesis that receptor mobile fraction and internalization are inversely related has thus been confirmed directly for the  $V_2$ -receptor. Comparable results for the  $V_1$ - and EGF-receptors are shown in Table II. Analysis of the pooled data (Tables I and II) for the 3 receptor systems yields a negative correlation ( $r = -0.95$ ) for  $f$  and receptor internalization.

Hillmann and Schlessinger [17] have proposed that EGF receptor lateral diffusion is mechanistically important in receptor endocytosis in that the EGF receptor must migrate to coated pit regions of the cell plasma membrane in order to undergo internalization (see also [22]). This introduces the interesting possibility, not only that receptor lateral mobility plays a role in signal transduction by bringing proteins into contact with one

Table II  
Summary of data for receptor internalization and fraction of immobilized receptors<sup>a</sup>

Receptor	Time after ligand addition (min)	$f$	Fraction of internalized receptors
$V_1$ -receptor [13]	10 (37°C)	0.36	0.75
$V_1$ -receptor [13]	10 (23°C)	0.50	0.51
EGF-receptor [15]	10 (37°C)	0.45	0.45
EGF-receptor [15]	30 (4°C)	0.90	0.15

<sup>a</sup> Analysis of the pooled data of  $f$  and fraction of internalized receptors for Tables I and II resulted in a correlation coefficient of  $-0.954$  ( $x$  intercept = 1.03,  $y$  intercept =  $-0.99$ ,  $n = 7$ )

another in the plasma membrane lipid bilayer, but that lateral diffusion of receptors may also be important in receptor down-regulation through endocytosis.

The EGF- [15,21,22,25], insulin- [26,27] and  $V_1$ -receptor [13,28–30] systems all display not only rapid activation of signal transduction, but also rapid desensitization of response concomitant with very rapid receptor internalization. The  $V_2$ -receptor shows both slower activation and down-regulation kinetics, concomitant with lower receptor lateral diffusion rates and slower receptor internalization kinetics. The persistence of a higher receptor mobile fraction, with the  $V_2$ -receptor moving more slowly to ultimate aggregation/immobilization/internalization and down-regulation than in the other systems mentioned, provides an explanation of the well-established amplification property of the adenylate cyclase system, i.e. that one ligand-receptor complex can activate many  $G_{sc}$  and subsequently adenylate cyclase molecules [6,31]. Receptor lateral diffusion could accordingly be a central mechanistic factor in G-protein-mediated signal transduction.

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